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Christine Todd Whitman, Administrator
Environmental Protection Agency
Ariel Rios Bldg. (1101A)
1200 Pennsylvania Ave. NW
Washington, DC 20460

Re: Comments on the HPV test plan for N-(1,1,3,3-tetramethylbutyl)-2-propenamide

Dear Administrator Whitman:

The following are comments on the test plan for N-(1,1,3,3-tetramethylbutyl)-2-propenamide, also known as tert-octylacrylamide (t-OAA; CAS no. 4223-03-4), apparently prepared by the National Starch and Chemical Co. for ICI Americas, Inc. These comments are submitted on behalf of People for the Ethical Treatment of Animals, the Physicians Committee for Responsible Medicine, the Humane Society of the United States, the Doris Day Animal League, and Earth Island Institute. These health, animal, and environmental protection organizations have a combined membership of more than ten million Americans.

It is unclear whether National Starch or a contract-testing laboratory and animal-breeding facility, Covance Inc., prepared the test plan. The National Starch representative who submitted the test plan is no longer with the company. If Covance is, in fact, responsible for the test plan, this represents a clear conflict of interest as this commercial laboratory also stands to profit from conducting the animal tests that it has recommended. National Starch and Chemical Co. and/or Covance proposes carrying out the following animal tests:

- OECD 203 for acute fish toxicity (40-120 fish killed)
- OECD 422, the "combined repeat-dose/reproductive toxicology screening test" (675 mammals killed)
- OECD 423 for acute mammalian toxicity (12 mammals killed)
- A "fertility test," identified without reference to an OECD test guideline; however, we presume this to be OECD 415 (1,300 mammals killed).

The total number of animals to be killed in the plan as currently presented is therefore extraordinarily high – more than 2,000. However, since OECD 422 covers reproductive toxicity, it is extremely difficult to imagine any result of OECD 422 that could necessitate an additional reproductive toxicity test in the form of OECD 415 in this screening level program. The authors of the test plan thus appear to be either confused about terminology or unaware and unconcerned that the testing proposal involves a great deal of redundancy that will lead to the suffering of an extremely large number of animals.

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Our objections to the proposed tests are as follows:

A. t-OAA should be included in a larger category

The HPV program contains several different branched alkane and alkene amides, and these could be included in a larger category, including n-dimethyloctanamide (CAS no. 1118929), N,N'-ethylenebis- octadecanamide (CAS no. 110305), oleamide (CAS no. 301020), and others. As we have pointed out many times in the past, important information about compounds covered by the HPV program is being lost due to the failure to compare compounds manufactured by different industries with similar structures and functional groups, as well as failing to consider their consistent structure-activity relationships. We urge National Starch to include this chemical in a larger category so as to reduce the number of animals killed in the HPV program.

B. The proposed fish testing is inappropriate for t-OAA

1. The t-OAA octanol/water partition coefficient may be too high for fish tests

The EPA states that fish tests are only appropriate if the log $K_{o/w}$ value of the test compound is less than 4.2 (EPA Federal Register, Dec. 26, 2000, p. 81695). It is quite possible that the log $K_{o/w}$ value of t-OAA is higher than 4.2, as it is a non-ionic organic compound that is prepared as a solution in "an appropriate solvent" (i.e. not water; test plan, p. 5). The test plan calls for determining the log $K_{o/w}$ value of t-OAA (p. 8), and it is therefore premature to propose a fish test until this value is known.

2. In vitro and in silico methods are available

As in our comments on more than 30 previous test plans submitted under the HPV program, we urge National Starch and Chemical Co. to use alternatives to the acute fish toxicity test, such as ECOSAR, TETRATOX, or the recently validated *Dar*T test. TETRATOX, an assay based on the protozoan *Tetrahymena pyriformis* (Larsen 1997), is an appropriate method for use in this plan. With 50% growth impairment as the endpoint, the results of this assay show close similarity to toxicity in the fathead minnow (Schultz 1997). The extensive available information demonstrates that TETRATOX is an effective alternative to fish testing. It is in fact already used extensively in industry, and is being considered for regulatory acceptance by the OECD. It is also rapid, easy to use, and inexpensive.

The recently validated *Dar*T test is another prospective replacement for *in vivo* tests. The test protocol and performance parameters are given in detail in Schulte (1994) and Nagel (1998). Briefly, however, the *Dar*T test uses fertilized zebrafish (*Danio rerio*) eggs as a surrogate for living fish. The exposure period is 48 hours, and endpoints assessed include coagulation, blastula development, gastrulation, termination of gastrulation, development of somites, movement, tail extension, eye development, circulation, heart rate, pigmentation and edema. Endpoints comparable to *in vivo* lethality include failure to complete gastrulation after 12 hours, absence of somites after 16 hours, absence of heartbeat after 48 hours, and coagulated eggs. The other endpoints provide further insight for a more detailed assessment of test

substances. The reliability and relevance of the *Dar*T test have recently been confirmed in an international validation study coordinated and financed by the German Environmental Protection Agency; predictions of acute toxicity from the *Dar*T test were highly concordant with *in vivo* reference data (Schulte 1996). This *in vitro* test has been accepted in Germany as a replacement for the use of fish in the assessment of wastewater effluent (Friccius 1995), and is clearly suitable for immediate use as a replacement for the use of fish in the HPV program's screening-level toxicity studies.

With respect to *in silico* methods, several quantitative structure-activity relationship (QSAR) programs for estimating toxicity to fish and other aquatic organisms are available. The EPA itself encourages the use of one established QSAR: ECOSAR (See EPA 2002a at http://www.epa.gov/oppt/newchems/21ecosar.htm).

3. *The ecologic relevance of fish toxicity should be taken into consideration*

The purpose of fish tests is not for predicting toxicity in individual fish, but for predicting economic loss (to commercial and "sport" fisheries) and ecologic damage (fish are an important part of the food chain). The test therefore aims to show whether pollution with t-OAA will result in large-scale fish death. However, water pollution can wipe out fish stocks even with no direct toxicity, because killing the food of the fish will lead to starvation. Carps and catfishes are herbivorous, eating mostly algae, whereas most other familiar North American freshwater fish species are carnivorous, eating worms, small crustaceans, smaller fish, insect larvae, etc. The toxicity of t-OAA towards these types of organism is unknown, as shown by the inclusion in the test plan of tests on an aquatic crustacean (*Daphnia*) and an alga (p. 9). Fish tests should not be carried out while other types of aquatic toxicity are unknown.

C. The mammalian toxicity testing proposed is inappropriate and redundant

The National Starch and Chemical Co. states that no data on acute toxicity are available (summaries, pp. 5-6). However, this very same company has carried out a single-dose mouse *in vivo* genetic toxicity study (summaries, p. 7, test plan, p. 10). Few details about the non-mutagenicity-related results of this study are provided, and no report of the study has been published, but doses of up to 700 mg per kilogram of body weight were administered, and the test plan states that t-OAA "induced signs of clinical toxicity in the treated animals and was cytotoxic to the bone marrow." Furthermore, a number of preliminary oral toxicity studies were carried out before this study (p. 10). It is crucial that the results of all these previously conducted studies be provided.

The existing acute toxicity data could be supplemented by *in vitro* data from a cytotoxicity assay. The most appropriate cytotoxicity assay involves assessing the cytotoxicity of compounds by measuring their effects on the viability of human basal keratinocytes. This viability is determined from the intensity of staining by neutral red (a dye), which is taken up by healthy cells more than by dead and low-viability cells. Furthermore, in the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC), a worldwide study organized by the Scandinavian Society for Cell Toxicology, basal cytotoxicity assays were found to be more reliable predictors of human lethal

doses, for 50 reference chemicals, than were rodent LD_{50} values (Clemedson 1996a, 1996b, 1998a, 1998b, 2000, Ekwall 1998a, 1998b, 2000). The EPA has issued a statement (http://www.epa.gov/chemrtk/toxprtow.htm) asking companies participating in the HPV program to use the human keratinocyte cytotoxicity assay as a supplement to the *in vivo* acute toxicity assay, especially for setting initial doses (2002b).

In the current information vacuum, discussion of other types of mammalian toxicity (chronic, subchronic, reproductive and developmental) is highly premature. Even most physicochemical data for t-OAA (e.g. vapor pressure, partition coefficients, solubility, hydrolysis) are absent. Data of these types are highly relevant to the approach that should be taken. Furthermore, meaningful discussion of hypothetical long-term, reproductive and developmental toxicity is difficult while absolutely no human exposure data are available. The test plan contains the following statement: "Human exposure is minimal throughout the manufacture of [t-OAA]" (p. 5). However, it provides no data to support this, and very little information about the controls and equipment used to limit human exposure.

If the National Starch and Chemical Co. insists that a developmental toxicity test is necessary at this stage, we strongly urge it to consider an *in vitro* method, in order to spare a large number of animals. An *in vitro* embryotoxicity test method, the rodent embryonic stem cell test, has recently been validated by the European Centre for the Validation of Alternative Methods, and ECVAM's Scientific Advisory Committee has concluded that this test is ready to be considered for regulatory purposes (Genschow 2002). This test is now commercially available in the U.S. We therefore urge the National Starch and Chemical Co. to consider the use of this *in vitro* test. If a positive result is found in the embryonic stem cell test, t-OAA should be treated as a development toxicant/teratogen, and no further testing should then be carried out within the screening-level program. Although we have written to the EPA repeatedly concerning the inclusion of the embryonic stem cell test in the HPV program, with correspondence dating back more than six months, we have received no reply. We urge the National Starch and Chemical Co. to correspond directly with the EPA on the incorporation of this validated non-animal test.

To summarize, the test plan appears to have been prepared with minimal understanding of the tests, and with little consideration as to either whether data are actually needed or to the sequence in which they will be needed. This test plan, therefore, represents a serious violation of the October 1999 agreement to reduce the number of animals killed in the HPV Program. That agreement stated, in part:

> (1) In analyzing the adequacy of existing data, participants shall conduct a thoughtful, qualitative analysis rather than use a rote checklist approach. Participants may conclude that there is sufficient data, given the totality of what is known about a chemical, including human experience, that certain endpoints need not be tested
>
> (8) … As with all chemicals, before generating new information, participants should further consider whether any additional information obtained would be useful or relevant.

We ask, again, that the EPA review this test plan with an eye towards the October 1999 agreement and thoughtful toxicology and ask National Starch to provide the necessary information prior to proposing more animal tests.

Thank you for your attention to these comments. I can be reached at 757-622-7382, extension 1304, or via e-mail at JessicaS@PETA.org.

Sincerely,

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Federal Agency Liaison
People for the Ethical Treatment of Animals

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